

structures. Thus the parameters could be optimized further according to the validation results.

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Observation of a Change in Twist of an RNA Kissing Complex Using the Angular Dependence of Fluorescence Resonance Energy Transfer

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Single-molecular-pair FRET is often used to study distance fluctuations of single molecules. It is harder to capture angular changes using FRET, because rotational motion of the dyes tends to wash out the angular sensitivity. Using a dye labeling scheme that minimizes the rotational motion of the dyes with respect to the RNA, we use spFRET to measure a change in twist angle of an RNA kissing complex upon protein binding. The model system studied here, R1inv-R2inv, is derived from the RNA I-RNA II complex in *E. coli*. RNA II is a primer for replication of the ColE1 plasmid; its function is modulated by interaction with RNA I. Rop protein is known to stabilize the bent R1inv-R2inv kissing complex against dissociation. The effect, if any, of Rop protein on the conformation of the kissing complex is not known. The eight minimized-energy NMR structures reported for R1inv-R2inv show a small difference in end-to-end distances and much larger difference in twist and bend angles. Our spFRET measurement showed an increase in average FRET upon Rop protein binding. With modeling, this increase could be attributed to a change in twist, but not bend or distance. The model used an MD simulation to calculate dye trajectories, and a direct integration of the resulting trajectories to predict FRET. The observed change in FRET is consistent with a decrease in the twist angle of the complex. We propose that Rop has a higher affinity for, and therefore serves to stabilize, these less stable (untwisted) conformations.

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Kinetic Destabilization through Single Point Mutations Emphasizes the Critical Role Played by Coupled Tertiary Interactions in the Folding of the Azoarcus Ribozyme

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Tertiary interactions stabilize RNA structure and impart function to RNAs. Using the *Azoarcus* group I ribozyme as a model system we determine the contributions of these interactions to folding of RNAs in general. Previous studies have taken a detailed thermodynamic look at key tertiary interactions for this ribozyme [1]. Here we make an effort to unravel the kinetic contributions of these tertiary interactions by abolishing them through single point mutations and studying the resulting dynamics through smFRET. We see that tertiary interaction mutants promote an increased number of excursions from the compact folded state of the ribozyme along with broadening the FRET population distribution. Our results show that the change in folding kinetics for each of the mutants is context dependent, providing us a kinetic stability map of the ribozyme's key tertiary interactions. A mutation in the P9 tetraloop results in the most "floppy" ribozyme, while a mutation in the P2 tetraloop causes only a small increase in transient unfolding, compared to the wild type ribozyme. Among the mutants, the folding rates show a smaller variation and are faster than the unfolding rates. These results show us how this long noncoding RNA has evolved to reduce conformational fluctuations as it proceeds in its folding path towards the native state.

[1] Behrouzi R., Roh J.H., Kilburn D., Briber R.M., Woodson S.A.; *Cell*, 2012, 149, 2, 348-357

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Local and Global Folding in a 58mer RNA Revealed by 2-Aminopurine Substitutions and Specific NMR Labels

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The ability of RNA to sample multiple conformations allows it to fulfill a myriad of biological roles. The folding pathways that RNA takes to achieve these conformations are not intuitive, and there is a pressing need to predictively model this folding behavior. The GTPase center (GAC) of the large subunit of the prokaryotic ribosome, is a well-studied 58mer that exhibits ion-dependent tertiary folding. By substituting the fluorescent base analog 2-aminopurine (2AP), ¹⁵N labeled bases, or ¹³C labeled bases in to specific loop and bulge regions we are able to monitor local changes that occur during Mg²⁺ induced tertiary folding. Being able to contrast the results from stopped flow kinetics, NMR dynamics and structural studies, we can see how local interactions make up tertiary folding. This work was funded by the NIH R01-GM098102 to KBH. Labeled RNA molecules were contributed by Agilent and NMR studies were supported by Agilent.

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RNA Junctions Structure and Distance Determination via Accurate Single-Molecule High-Precision FRET Measurements

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Förster-Resonance-Energy-Transfer (FRET) restrained high-precision structural modeling is a powerful tool for analyzing biomolecular structures. Here we apply multi-parameter fluorescence detection (MFD) of single molecules and ensemble Time-Correlated Single Photon Counting measurements to perform FRET study on RNA three- and four-way-junctions (4WJs and 3WJs) with a high level of precision in distance better than 1% of the Förster radius [1]. We have generated a database of RNA 4WJs and six different RNA 3WJs with different bulges and sequences to study the influence of these factors on the junction conformations of RNA 3WJs. Overall 260 FRET pairs were measured with single-molecule MFD at 20 mM MgCl₂ concentration and analyzed with the analysis toolkit [2] that includes probability distribution analysis (PDA) for FRET distance determination and FRET position and screening (FPS) toolkit for structural model generation.

Monte Carlo simulations showed that sterically allowed conformational space for RNA junctions is large. However, FRET measurements detect the existence of three different static conformers for RNA 4WJ, whereas RNA 3WJs have only one predominant static conformation. sterically allowed conformational space for RNA is large. Their junction geometry was described in terms of mutual and Euler angles between helices. The FRET-derived structures suggest that the sequence dictates a junction specific conformation within the large topology space. Furthermore we see that bulges in the junction region determine orientation and rotation of helices and induce coaxial stacking between two of them.

[1] Antonik, M., et al., *J.Phys.Chem.B*, 110, 6970-6978 (2006)

[2] Kalinin, S. et al, *Nat. Meth.*, 9, 1218-1225 (2012)

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RNA Pseudoknot Folding Energy Landscape Elucidated with T-Jump Measurements and Kinetic Modeling

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Pseudoknots are minimal tertiary motifs in RNA that are involved in many biological functions determined by their structure, stability and dynamics. They also stabilize the structures of several ribozymes and are often the rate-limiting step in their folding pathways. Although many studies have reported on pseudoknot stability, and theoretical and computational studies have proposed folding pathways for some pseudoknots, there are few experimental studies on pseudoknot folding kinetics. Therefore, a complete picture of how pseudoknots fold to achieve their functional structure is lacking.

Here, we report folding kinetics of the VPK pseudoknot, a variant of the Mouse Mammary Tumor Virus (MMTV) pseudoknot, which is involved in ribosomal frameshifting. We combine rapid temperature-jump (T-jump) with time-resolved fluorescence spectroscopy, and global analysis of equilibrium and kinetics measurements, to elucidate the folding energy landscape. We use 2AP (a fluorescent analog of adenine) placed at different positions along the RNA sequence as a probe of RNA conformations, both under equilibrium conditions and in response to the T-jump perturbation. We also, independently, measure the equilibrium and folding kinetics of two hairpin structures that are believed to be intermediates in the folding pathways. The complete set of equilibrium and kinetics measurements on the pseudoknot and the two hairpins are described in a self-consistent manner in terms of a 4-state kinetic model and a minimal set of parameters. Our results provide the first experimental evidence of multiple parallel folding/unfolding pathways in RNA pseudoknots, as indicated by previous simulations studies.

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A Biophysical Analysis of the CDK5R2 mRNA G-Quadruplex Secondary Structure and its Role in the Pathogenesis of Fragile X Syndrome

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In silico energy modeling has shown that guanine-rich regions of certain nucleic acids are capable of forming secondary structures known as G-quadruplexes. These structures are subject to unique protein binding and therefore partake in gene regulation by mediating translation of messenger RNA (mRNA). Such regulation suggests that G-quadruplex-containing mRNAs